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Rapid Degradation of AU-Rich Element (ARE) mRNAs Is Activated by Ribosome Transit and Blocked by Secondary Structure at Any Position 5′ to the ARE

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The 3′ noncoding region (NCR) AU-rich element (ARE) selectively confers rapid degradation on many mRNAs via a process requiring translation of the message. The role of cotranslation in destabilization of ARE mRNAs was examined by insertion of translation-blocking stable secondary structure at different sites in test mRNAs containing either the granulocyte-macrophage colony-stimulating factor (GM-CSF) ARE or a control sequence. A strong (−80 kcal/mol [1 kcal = 4.184 kJ]) but not a moderate (−30 kcal/mol) secondary structure prevented destabilization of mRNAs when inserted at any position upstream of the ARE, including in the 3′ NCR. Surprisingly, a strong secondary structure did not block rapid mRNA decay when placed immediately downstream of the ARE. Studies are also presented showing that the turnover of mRNAs containing control or ARE sequences is not altered by insertion of long (1,000-nucleotide) intervening segments between the stop codon and the ARE or between the ARE and poly(A) tail. Characterization of ARE-containing mRNAs in polyadenylated and whole cytoplasmic RNA fractions failed to find evidence for decay intermediates degraded to the site of strong secondary structure from either the 5′ or 3′ end. From these and other data presented, this study demonstrates that complete translation of the coding region is essential for activation of rapid mRNA decay controlled by the GM-CSF ARE and that the structure of the 3′ NCR can strongly influence activation. The results are consistent with activation of ARE-mediated decay by possible entry of translation-linked decay factors into the 3′ NCR or translation-coupled changes in 3′ NCR ribonucleoprotein structure or composition.

Short-lived mRNAs are typically those encoded by genes that respond rapidly and transiently to a variety of extracellular stimuli, which include cytokines, lymphokines, and proto-oncogenes. The short half-life of many cytokine mRNAs, such as that for granulocyte-macrophage colony-stimulating factor (GM-CSF), is largely conferred by an AU-rich element (ARE) found in the 3′ noncoding region (NCR), corresponding to a repeated consensus sequence, AUUUA (48). Recent mutagenic analysis demonstrated that the minimal functional ARE motif is UUAUUUAUU (63) or the related sequence UUA UUA(U/A)(U/A) (30). The destabilizing elements of most proto-oncogene mRNAs are more complicated than those of cytokine messages (reviewed in reference 43). For instance, the c-*fos* mRNA appears to utilize at least two functionally interdependent instability determinants in addition to the ARE: one within the 3′ end of the coding region, and another represented by a poly(U)-rich sequence in the 3′ NCR (10, 11, 21, 41, 49, 50, 54, 56, 60). Although degradation of the GM-CSF mRNA is functionally simpler than that of c-*fos* mRNA, an additional element located upstream of the GM-CSF ARE prevents destabilization in response to mitogens (20).

Little is understood of the mechanisms by which AREs target mRNAs for selective destabilization. It is known that rapid mRNA turnover mediated by GM-CSF or c-*fos* destabilizing elements occurs predominantly in the cytoplasm (1, 46, 48, 50) and can be transiently stabilized by treatment of cells with protein synthesis inhibitors (48) or calcium ionophores (58). Several AU-rich sequence-binding proteins (AUBPs), originally identified by in vitro UV cross-linking (5, 6, 15, 53), are thought to influence stabilization and destabilization of ARE mRNAs. Binding by a 30-kDa cytosolic T-cell-specific AUBP correlates with mRNA destabilization (6), whereas binding by an inducible 32-kDa AUBP (termed AUBF) correlates with mRNA stabilization (15, 42). Binding by AUBF has been found to protect against rapid mRNA degradation in an in vitro decay extract with polysomal mRNAs (42), implicating it as a potential masking factor that might initiate mRNA degradation upon displacement. A 40-kDa cytosolic AUBP termed AUF1 specifically binds c-*myc* and GM-CSF AREs in vitro and may participate in rapid degradation of these messages (8, 65). The possible roles of AUBPs in vivo are not known, however, and several may correspond to heterogeneous nuclear ribonucleoprotein particle (hnRNP) A1 and C proteins, which bind U-rich sequences and participate in transport of mRNAs (18, 22).

A number of studies have causally linked accelerated mRNA turnover with translation in eukaryotes. For several mRNAs, the link is indirect, since inhibition of protein synthesis in *trans* prevents destabilization (2, 44). In contrast, destabilization of many mRNAs, particularly but not exclusively those containing AREs, requires translation of the mRNA to "activate" the destabilization element (1, 3, 9, 16, 31, 35, 38, 46, 47, 57). Destabilization of c-*fos* and c-*myc* mRNAs in mammalian cells is facilitated cotranslationally by elements in the coding region and 3′ NCR (3, 47, 49, 54, 57). The GM-CSF ARE is also activated cotranslationally (1, 46, 57). Evidence for cotranslational mRNA decay mediated by the GM-CSF ARE indicates that rapid mRNA turnover could be inhibited by insertion of ribosome-blocking secondary structure adjacent to the cap (1), by deletion of translation initiation codons (46), or by dissociation of the mRNA from polysomes using the translation-blocking iron-responsive element (57). The stabilization of ARE mRNAs by cap-proximal secondary structure was shown

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to result from the inability to associate with ribosomes rather than by direct inhibition of a 5′ → 3′ nuclease, since insertion of an internal ribosome entry site downstream of the secondary structure restored both translation and rapid degradation of the mRNA (1). Translation of the mRNA therefore plays a direct role in triggering rapid degradation controlled by many destabilizing elements, but the process that links translation to activation of mRNA turnover is not clear.

Translation of short-lived mRNAs could activate degradation in several ways. Some evidence suggests that translation, deadenylation, and rapid turnover of certain short-lived mRNAs may be related. Short-lived ARE-containing mRNAs for c-*fos*, c-*myc*, and tumor necrosis factor are rapidly deadenylated prior to degradation (7, 31, 32). Moreover, translation has been found to increase the rate of poly(A) shortening (31, 51, 55, 56), suggesting that one potential function of the ARE might be to enhance the rate of cotranslational deadenylation. On the other hand, rapid deadenylation of the short-lived yeast *MFA2* mRNA is not controlled cotranslationally, and nonsense-mediated turnover of the *PGK1* mRNA is not coupled to rapid deadenylation (17). In addition, mutant c-*fos* mRNAs can be deadenylated in mammalian cells without rapid degradation of the mRNA body (4, 49). Thus, while an increased rate of deadenylation may be linked to mRNA translation and AREs may accelerate poly(A) removal in at least some mRNAs, loss of poly(A) is not always sufficient to initiate accelerated decay. Other models have been proposed to account for the link between translation, rapid mRNA decay, and activation of destabilization elements (reviewed in reference 43). In general, these models propose that the transiting ribosome activates the destabilizing element either by direct contact or indirectly. Direct contact between ribosomes and destabilizing elements (most are in the 3′ NCR) might remove element-specific masking proteins and/or deposit element-specific factors that promote rapid mRNA degradation. Indirect promotion of mRNA degradation by ribosomes might occur by alteration of RNP structure or composition, perhaps making the destabilizing element vulnerable to recognition by the decay machinery.

In this study, we demonstrate that cotranslational destabilization of mRNAs containing the GM-CSF ARE is blocked by strong secondary structure inserted at any position 5′ to the ARE, including several hundred nucleotides downstream from the translation termination codon in the 3′ NCR. In contrast, secondary structure inserted 3′ to the ARE does not block rapid mRNA degradation. Rapid mRNA turnover is shown to require translation of the coding region and a second step in which access to the 3′ NCR is essential for a 5′ → 3′ processive event.

MATERIALS AND METHODS

Plasmid construction. All constructs were derived from plasmids pLSAT and pLSGC, described previously (1). Plasmids contain the adenovirus major late promoter, tripartite leader 5′ NCR, and hepatitis B virus surface antigen (HBsAg) coding region. pLSAT contains the GM-CSF ARE, β-globin 3′ NCR, and polyadenylation signal (48). pLSGC contains a mutated ARE sequence in which G and C residues have been interspersed (48), constructed in the same genetic background as pLSAT. Plasmids pLSGC-5′Bam and pLSAT-5′Bam were constructed by digestion at the unique *Sal*I site, which is located 12 nucleotides (nt) upstream of the HBsAg AUG and at the 3′ end of the tripartite leader, of parent plasmids pLSGC and pLSAT, which was followed by Klenow repair. Plasmid pLSAT-Bam-ORF was constructed by digestion of pLSAT at the *Xba*I site in the HBsAg coding region and Klenow repair. *Bam*HI linkers (12 bp) were subjected to prior ligation and then ligated to the repaired sites. Plasmids acquiring *Bam*HI sites were identified by restriction enzyme analysis and confirmed by DNA sequencing, and clones possessing five copies of linkers were used for further study. Plasmid pLSAT-Bam-3′ARE was constructed by digestion of pLSAT at the unique *Eco*RV site located ~55 nt downstream of the ARE, followed by ligation to *Bam*HI linkers and identification of positive clones as described above. Plasmid pLSAT-3′Bam was constructed by first subcloning the 660-bp *Nhe*I fragment containing the 3′NCR ARE from pLSAT into the pSK Bluescript vector (1) and then digesting the resulting construct (pMAT) at a unique *Pst*I site located 75 nt upstream of the ARE, followed by ligation to *Bam*HI linkers. Positive clones were identified and sequenced, the *Nhe*I fragment was reconstructed into pLSAT, and clones were screened for the correct orientation. Plasmids pLSAT-Bam-stop and pLSAT-stop-Bam were constructed by first introducing a *Bam*HI linker between the third nucleotide of the penultimate codon and the stop codon or immediately 3′ to the third nucleotide of the stop codon. Insertions were generated by PCR with the inside-outside priming technique and verified by DNA sequence analysis.

Cell culture and DNA transfections. 293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% supplemented calf serum, 2 mM L-glutamine, and 100 μg of gentamicin per ml. Transfection of 10-cm plates of 293 and HeLa cells was performed in triplicate by the calcium phosphate precipitation technique with 3 μg of test plasmid and 1 μg of a plasmid expressing β-galactosidase as a control for transfection efficiencies. Cells were harvested after 48 h, and transfection efficiencies were determined by β-galactosidase assays as described previously (1). Transfection efficiencies averaged 30% (±10%). Plates of cells deviating in transfection efficiency by more than 10% from the mean were discarded.

Analysis of protein and mRNA levels. Levels of HBsAg protein synthesis were determined in cells labeled with [^{35}S]methionine. Labeling of cells, preparation of extracts, immunoprecipitation of HBsAg, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography were carried out as described previously (1). RNAs were isolated by guanidine HCl extraction followed by centrifugation through a CsCl cushion (45). Northern (RNA blot) analysis was performed with equal amounts of whole cytoplasmic or polyadenylated [poly(A)$^+$] mRNA in formaldehyde-agarose gels. The mRNAs were transferred to a Nytran membrane and hybridized to ^{32}P-labeled probes prepared from the HBsAg coding region by the random primer method (13). Levels of mRNAs were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA present. Samples were rerun if the amount of hybridized GAPDH mRNA varied between lanes by more than twofold. Quantitation was obtained by laser densitometry of autoradiographs.

RT-PCR. In the reverse transcription-PCR (RT-PCR) assay, equal amounts of whole cytoplasmic or poly(A)$^+$ RNAs were analyzed. Whole cytoplasmic RNA (15 μg) was heated to 65°C for 3 min, quenched on ice, and used for RT-PCR. Reactions were carried out in 20-μl volumes containing 30 pmole of 3′ NCR oligonucleotide DNA primer in PCR buffer (50 mM Tris-HCl [pH 8.5], 6 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 1 mM deoxynucleoside triphosphate mix, 20 U of RNasin [Promega Biotech, Madison, Wis.], 10 U of avian myeloblastosis virus RT) at 41°C for 2 h. RT was inactivated at 95°C for 10 min. PCR was carried out directly by supplementing reaction mixes in a final volume of 80 μl with 30 pmol of S-antigen coding region oligonucleotide DNA primer and 2.5 U of *Taq* polymerase. PCR was performed for 25 cycles with a step program (94°C for 1 min, 50 to 60°C for 3 min, and 72°C for 3 min) followed by a 15-min final extension at 72°C. PCR products were resolved by 1.5% agarose gel electrophoresis and photographed.

RESULTS

Ribosomes must transit the entire coding region to activate the GM-CSF ARE. We first examined the extent to which ribosome transit of the mRNA is required to initiate rapid degradation controlled by the GM-CSF ARE. The experimental mRNAs contain the adenovirus tripartite leader 5′ NCR, the HBsAg (S antigen) coding region, and the GM-CSF ARE (LSAT) or a GC control sequence (LSGC) in the 3′ NCR (1, 48) (Fig. 1). Stable secondary structures were created by ligating five copies of a 12-nt-long *Bam*HI linker, which were inserted at various positions in the short-lived test mRNA (LSAT series) and in the long-lived counterpart (LSGC series). The estimated free energy of these structures ($\Delta G = -80$ kcal/mol [1 kcal = 4.184 kJ]) is in considerable excess of that required to block the transit of 80S ribosomes and 40S ribosomal subunits ($\Delta G > -40$ kcal/mol) (26, 29, 37). Secondary structure was inserted immediately upstream of the initiation codon, creating LSAT-5′Bam and LSGC-5′Bam (Fig. 1). Placement of secondary structure at this site allows 40S ribosomal subunits to bind mRNA but should block the formation of 80S elongating ribosomes, thereby testing whether binding of 40S ribosomal subunits is sufficient to trigger destabilization. A stable stem-loop structure was also inserted 87 nt downstream from the initiation codon to create the mRNA LSAT-

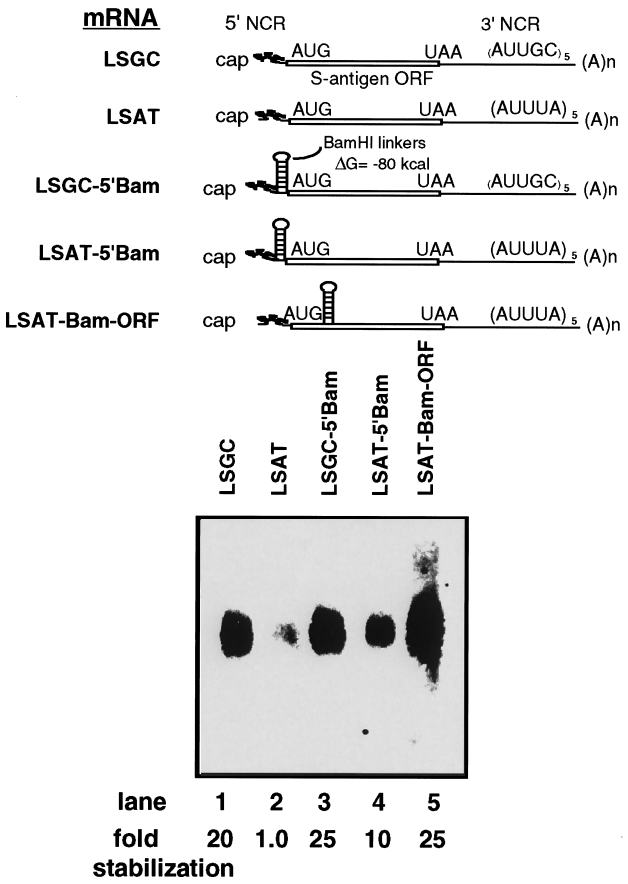

FIG. 1. Stability of mRNAs containing secondary structure immediately upstream or downstream of the AUG. HeLa cells were transfected with plasmids, transfection efficiencies were monitored, and poly(A)$^+$ mRNA was prepared 48 h later. Equal amounts of mRNA were subjected to Northern analysis in formaldehyde-agarose gels as described previously (1). After transfer to nitrocellulose membranes, blots were hybridized to ^{32}P-labeled probes prepared from the HBsAg (S antigen) coding region. Data acquisition and quantitation were performed by autoradiography and scanning densitometry, and data are represented as fold stabilization over that of the unstable control mRNA LSAT. 5′ NCR, curved line; 3′ NCR, straight line; S antigen open reading frame (ORF), box; and secondary structure created by ligating multiple copies of *Bam*HI linkers, hairpins. Calculation of the free energy of RNA secondary structures was performed with standard thermodynamic values (14).

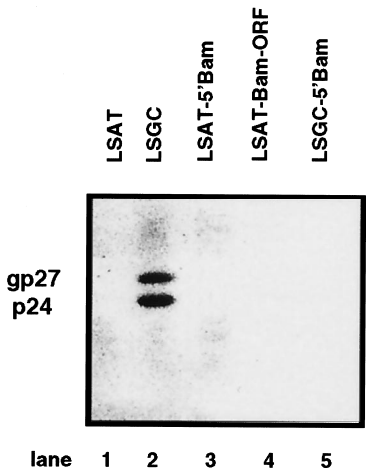

FIG. 2. Translation of mRNAs containing stable stem-loop structures upstream or downstream of the AUG. Transfection of cells was carried out as described in the legend to Fig. 1. Translation efficiencies were determined in transfected cells by immunoprecipitation of S antigen from [^{35}S]methionine-labeled cell lysates with equal amounts of protein (equal counts per minute), followed by SDS-PAGE and autoradiography (1). Glycosylated (27 kDa) and nonglycosylated (24 kDa) S antigen polypeptides are synthesized, as indicated.

Bam-ORF (Fig. 1). This mutant blocks translation at codon 29 (~15% of the coding region [Fig. 2, lane 4]). Translation of this mRNA produces a protein product that is too small to be detected by antibodies to S antigen, but the LSAT-Bam-ORF mRNA sedimented in gradients with polysomes (data not shown), indicating that it was associated with ribosomes. The LSAT-Bam-ORF mRNA will therefore determine whether degradation is induced by prematurely terminating translation, as observed for β-tubulin and nonsense mRNAs (39, 52, 60). Secondary structure was also inserted immediately flanking the upstream (LSAT-Bam-stop) or downstream (LSAT-stop-Bam) side of the stop codon (Fig. 3). These mutants will determine whether activation of the GM-CSF ARE requires ribosome elongation through the complete coding region, as described for unstable c-*fos* mRNA (54). Finally, several mRNAs were constructed to assess whether the 3′ NCR itself plays a role in cotranslational activation of the ARE. A stable stem-loop structure was inserted in the 3′ NCR, 125 nt downstream of the stop codon and 80 nt upstream of the GM-CSF ARE (LSAT-3′Bam) or 55 nt downstream of the ARE (LSAT-Bam 3′ARE). Thus, a roughly continuous series of mRNA variants containing stable secondary structure, progressing from upstream of the initiation codon, through the coding region, and into the 3′ NCR between the site of translation termination and the ARE or the ARE and the poly(A) tail were constructed.

The steady-state levels of short- and long-lived poly(A)$^+$ mRNAs containing stable hairpins were examined by Northern blot analysis to determine the relative half-lives of the mRNAs. It was shown previously that the rapid turnover of the parental mRNAs containing the GM-CSF ARE (LSAT and its variants) occurs in the cytoplasm and that the cytoplasmic steady-state level determined by Northern blot analysis accurately corresponds to the actual half-life (1). Therefore, it was not necessary to separately determine mRNA half-lives in this study. Translation was blocked by insertion of secondary structure immediately upstream of the initiating AUG in the stable control mRNA (LSGC-5′Bam [Fig. 2, lanes 2 and 5]) without a change in cytoplasmic abundance (Fig. 1, lanes 1 and 3). All mRNAs in this study used as a reporter the HBsAg coding region, which gives rise to 24-kDa (p24) and glycosylated 27-kDa (gp27) polypeptides. LSGC and LSGC-5′-Bam mRNAs were found associated with polysomes (data not shown), excluding the possibility that secondary structure stabilized the mRNAs by preventing their association with ribosomes. In contrast, insertion of secondary structure in an identical location in the normally unstable LSAT transcript caused a 10-fold increase in its cytoplasmic abundance (Fig. 1, lanes 2 and 4; compare LSAT with LSAT-5′Bam). These results indicate that the binding of 40S ribosomal subunits to an mRNA containing the GM-CSF ARE is not sufficient to induce its degradation. Insertion of secondary structure 29 codons downstream from the initiating AUG of unstable LSAT mRNA (LSAT-Bam-ORF) also caused stabilization of the message (Fig. 1, lane 5). Therefore, assembly of initiating 80S ribosomes and their partial transit through the coding region are not sufficient to trigger selective mRNA decay.

Rapid mRNA turnover mediated by the c-*fos* destabilizing elements requires translation of the c-*fos* coding region, including transit of ribosomes into the 3′ terminus of the coding

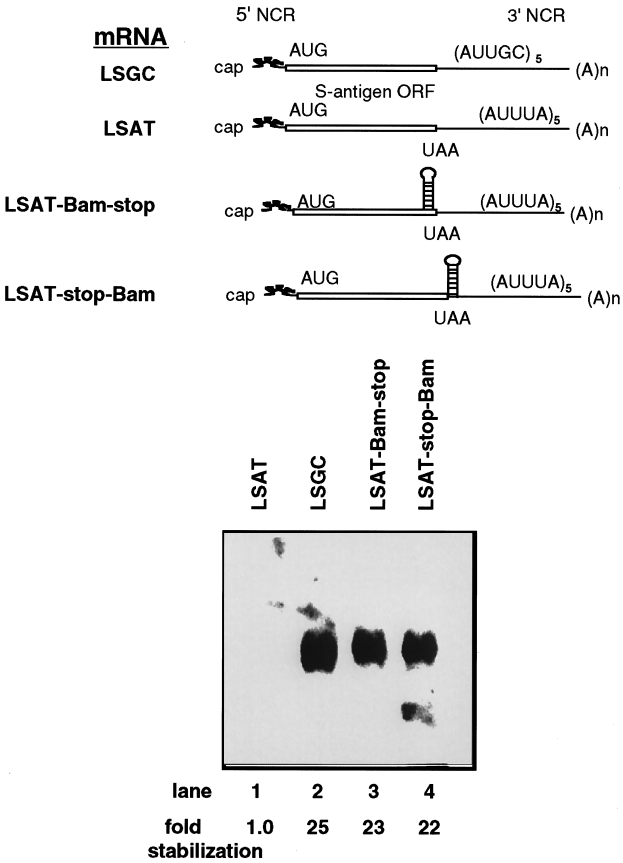

FIG. 3. Stability of mRNAs containing secondary structure flanking the translation termination codon. Transfection of HeLa cells and Northern blot analysis of cytoplasmic poly(A)$^+$ test mRNAs were carried out as described in the legend to Fig. 1. Stable stem-loop structures were inserted adjacent to the stop codon. Quantitation is represented as the fold difference in stabilization over that of the short-lived LSAT mRNA, which is visible in overexposed gels.

region (54). To assess whether the GM-CSF ARE has similar requirements for induction of mRNA destabilization, two variant mRNAs were constructed, one containing stable secondary structure between the penultimate codon and the stop codon (LSAT-Bam-stop), and another containing stable secondary structure 8 nt downstream of the stop codon (LSAT-stop-Bam). Strong secondary structure in these positions should prevent ribosomes from translating through the 3′ terminus of the coding region. If mRNA decay is activated by ribosome entry only into the 3′ terminus of the coding region, then either or both of the variant mRNAs should be degraded. If translation must proceed through the entirety of the coding region to induce destabilization, perhaps propagating alterations to the 3′ NCR, then both variants should be blocked in rapid turnover. The steady-state cytoplasmic level of poly(A)$^+$ mRNAs was examined by Northern analysis (Fig. 3). Surprisingly, both LSAT variant mRNAs were stabilized against rapid turnover. The smaller band evident in lane 4 was not observed in other experiments and is therefore considered not significant. Activation of the GM-CSF ARE and rapid mRNA degradation therefore involve a mechanism in which the mRNA must first be fully translated past the 3′ terminus of the coding region to induce degradation.

Destabilization induced by the ARE is blocked by strong secondary structure in the 3′ NCR. Several additional mRNAs were constructed to assess whether destabilization requires that the 3′ NCR participate in activating destabilization through a process linked to translation. For example, this mechanism could correspond to movement of ribosomes, subunits, or translation-associated decay factors into the 3′ NCR (possibly contacting the ARE) or propagation of structural or compositional alterations to the 3′ NCR RNP. Stable secondary structure was inserted 125 nt downstream from the translation termination codon and ~80 nt upstream of the ARE (variant LSAT-3′Bam) or 55 nt downstream of the ARE (LSAT-Bam3′ARE). Surprisingly, secondary structure inserted between the stop codon and ARE (LSAT-3′Bam) prevented degradation of the mRNA, whereas secondary structure inserted between the ARE and poly(A) tail did not (Fig. 4A). Thus, activation of the GM-CSF ARE is blocked by secondary structure at any position upstream but not downstream of the ARE. It was then determined whether secondary structure inserted in the 3′ NCR prevented translation of the mRNA, accounting for stabilization of those mRNAs with the ARE. Cells were transfected with plasmids expressing LSAT, LSGC, LSAT-3′Bam, and LSAT-Bam3′ARE mRNAs and labeled with [^{35}S]methionine, and reporter protein S antigen levels were analyzed by immunoprecipitation and SDS-PAGE (Fig. 4B). It is evident that insertion of secondary structure between the stop codon and ARE stabilized the mRNA but did not prevent its translation. The stable ARE-containing mRNA LSAT-3′Bam is translated to almost the same level as the stable control mRNA LSGC (Fig. 4B, compare lanes 2 and 3). These results indicate that in addition to translation of the coding region, activation of the GM-CSF ARE also involves a 5′ → 3′ processive event that includes the 3′ NCR.

Studies were next conducted to provide some measure of the strength of the 5′ → 3′ process that triggers mRNA destabilization in the 3′ NCR. Another set of mRNAs containing either three or five *Nhe*I linkers, inserted 125 nt upstream of the ARE (LSAT-3′Nhe3 and LSAT-3′Nhe5 [Fig. 5]) were constructed. The *Nhe*I linkers form moderately stable structures (ΔG = −20 to −30 kcal/mol), which can be melted by elongating ribosomes (26, 29, 37). The LSAT-3′ Nhe variants therefore provide a quantitative assessment of the amount of secondary structure required to block degradation when inserted upstream of the ARE. Northern analysis of cytoplasmic poly(A)$^+$ mRNAs showed that the LSAT-3′ Nhe variants were almost as unstable as the short-lived LSAT control mRNA (Fig. 5A), demonstrating only a twofold increase in stability. LSAT-3′ Nhe mRNAs were associated with polysomes (data not shown), which was shown directly by immunoprecipitation of [^{35}S]methionine-labeled cell extracts (Fig. 5B). These data demonstrate that small amounts of the S antigen reporter protein could be detected in cells expressing LSAT-3′ Nhe and LSAT mRNAs (~2 to 5% of the level in cells expressing LSGC mRNA).

Studies were performed to compare the relative stabilities of secondary structures formed from multimerized *Bam*HI and *Nhe*I linkers (Fig. 6). Whole cytoplasmic RNAs were prepared from transfected cells, and S antigen RNAs were reverse transcribed by priming with a DNA oligonucleotide in the 3′ NCR at a position downstream of the location of inserted secondary structure (Fig. 6). cDNAs were then amplified by PCR priming with 3′ and 5′ DNA oligonucleotides flanking the site of inserted secondary structure, and products were resolved by agarose gel electrophoresis. LSGC mRNA produced a DNA fragment migrating at 595 bp, and LSAT-3′ Nhe5 mRNA produced a 635-bp fragment, as expected. LSAT-3′Bam mRNA did not generate a detectable product even after gels were blotted with a ^{32}P-labeled probe (data not shown). If the *Bam*HI structure was not a barrier to RT-PCR, a 655-bp DNA fragment would

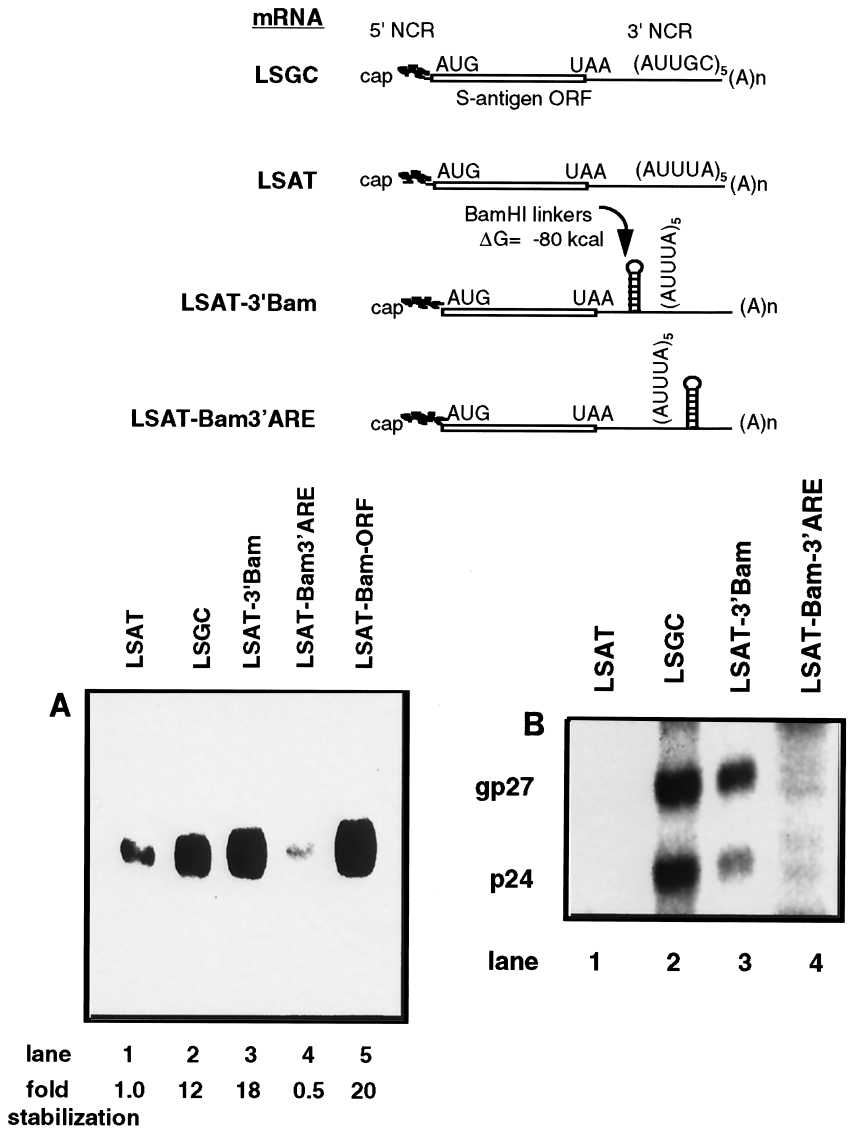

FIG. 4. Stability of mRNAs containing secondary structure in the 3′ NCR flanking the ARE. HeLa cells were transfected with plasmids, and transfection efficiencies were monitored. (A) Northern analysis of poly(A)$^+$ mRNA prepared 48 h after transfection. Equal amounts of mRNA were subjected to Northern analysis in formaldehyde-agarose gels. After transfer to nitrocellulose membranes, blots were hybridized to ^{32}P-labeled probes prepared from the S antigen coding region. (B) Translation analysis of mRNAs. Transfected cells were labeled with [^{35}S]methionine, and equal amounts of cell extracts were immunoprecipitated with antibodies to S antigen. Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. Quantitation was performed by scanning densitometry, and data are represented as fold stabilization over that of the unstable control mRNA LSAT.

have been synthesized. Co-RT-PCR of LSGC and LSAT-3′Bam mRNAs produced a single product corresponding to the unstructured (LSGC) mRNA (data not shown). These data therefore indicate that the multimerized *Bam*HI secondary structure ($\Delta G = -80$ kcal/mol), which blocks ARE-mediated cotranslational degradation, is also a strong barrier to processivity by RT-PCR. However, the weaker *Nhe*I structure ($\Delta G = -30$ kcal/mol) does not block rapid turnover of ARE mRNAs and does not prevent RT-PCR processivity.

Characteristics of the decay of the short-lived ARE-containing mRNAs. The results presented above indicate that placement of strong secondary structure only a short distance 5′ to the ARE apparently blocks rapid mRNA turnover, whereas secondary structure 3′ to the element does not. However, because steady-state levels of poly(A)$^+$ RNAs were examined, it is also possible that stable mRNAs that were either deadenylated or degraded in a 3′ → 5′ direction to the site of inserted secondary structure might accumulate. Whole cytoplasmic RNA from cells transfected with plasmids expressing stable LSGC or LSAT-3′Bam or unstable LSAT or LSAT-Bam3′ ARE mRNAs was therefore analyzed. If the body of the LSAT Bam-3′ARE mRNA is not fully degraded but instead only deadenylated, it should give rise to an RNA fragment similar in amount to and only slightly smaller than the stable LSGC mRNA. If the mRNA body is degraded to the site of secondary structure and stable intermediates accumulate, smaller RNA fragments might be observed. Only low levels of full-length LSAT and LSAT-Bam-3′ ARE mRNAs (secondary structure 3′ to the ARE) were found, identical to those observed in the poly(A)$^+$ fraction (Fig. 7). The long-lived ARE mRNA, which was stabilized by secondary structure between the stop codon and the ARE (LSAT-3′Bam), was as abundant as the LSGC

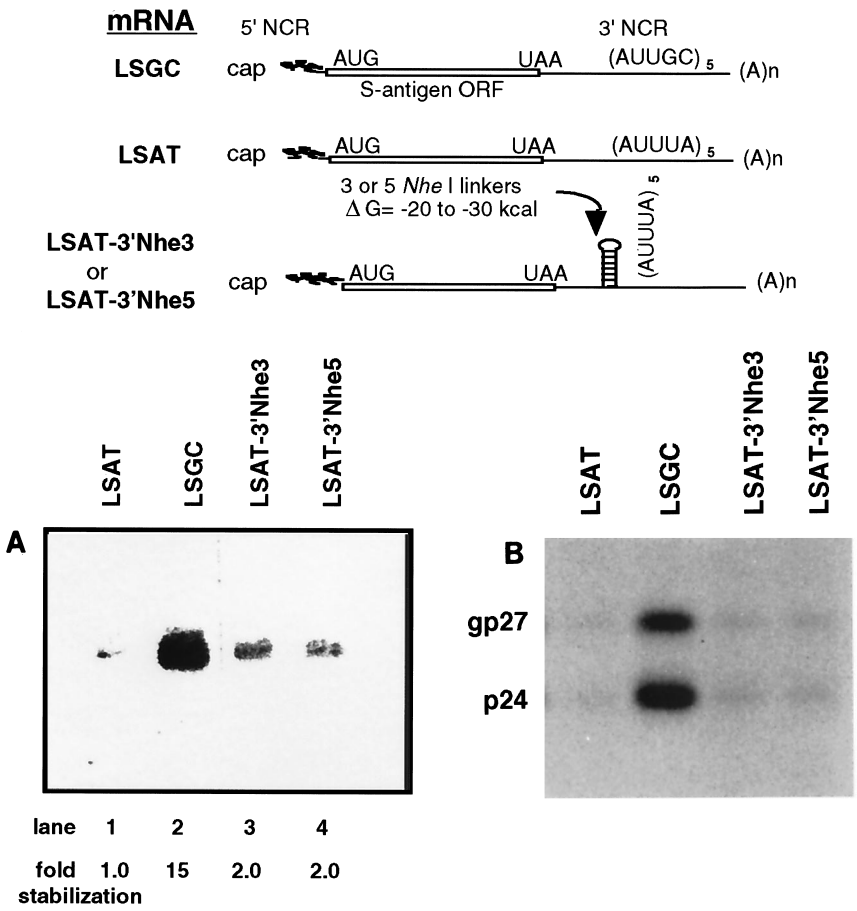

FIG. 5. Stability of mRNAs with moderately stable secondary structure in the 3′ NCR. (A) Northern analysis of poly(A)$^+$ mRNAs. (B) Immunoprecipitation of [^{35}S]methionine-labeled cell extracts with antibodies to S antigen. Quantitation of autradiographs was carried out as described in the legend to Fig. 4. LSAT-3′Nhe3 and LSAT-3′Nhe5 mRNAs contain three and five *Nhe*I linkers, respectively, inserted in the 3′ NCR at the same sites as the *Bam*HI linkers in LSAT-3′Bam mRNA. Fold stabilization represents the fold increase over that of LSAT mRNA in overexposed gels.

control and of similar size. These results indicate that mRNAs containing secondary structure 3′ to the GM-CSF ARE were rapidly degraded and not just deadenylated. Furthermore, no degradation intermediates were found to accumulate for unstable ARE mRNAs digested from either the 5′ or 3′ direction to the site of inserted secondary structure.

Distance between the stop codon and the ARE is not a critical feature of accelerated mRNA decay. The results described above are consistent with the GM-CSF ARE activation of decay via a mechanism that requires a processive transit of ribosomes through the coding region and access of the 3′ NCR to either signals or factors that move in a 5′ → 3′ direction. It is therefore possible that the distance between the ARE and the translation termination codon might be an important regulator of accelerated mRNA decay. Unfortunately, because it is not known whether or where ribosomes actually dissociate from the mRNA in vivo after termination of translation (reviewed in reference 19), it cannot be known whether ribosomes might directly contact and activate the ARE. In the unstable LSAT mRNA, the GM-CSF ARE is located 205 nt downstream from the stop codon, and the intervening segment contains no AUGs in good codon context for reinitiation, which is an exceedingly rare event under any condition (23, 36). Basic studies were therefore carried out to determine whether the distance between the ARE and the stop codon influences the rate of decay, possibly by juxtaposing the terminating translation apparatus with the destabilizing element. mRNAs in which the intersegmental distance between the stop codon and the ARE was increased from 205 to 1,095 nt by insertion of an 890-nt fragment were constructed (LSAT-890; see Materials and Methods for details). This RNA segment is devoid of initiation codons and sequences that might act as destabilizing elements, such as U-rich or AU-rich motifs. A similar mRNA was constructed for the stable LSGC mRNA as well. Northern blot analysis of poly(A)$^+$ cytoplasmic mRNA showed that this intervening sequence did not increase the stability of ARE-containing mRNAs (Fig. 8). A second variant in which the 890-nt segment was inserted between the ARE and poly(A) tail (LSAT-890 3′) was as unstable as the LSAT control. In LSAT mRNA, ~80 nt normally separate the ARE from the poly(A) tail. The cytoplasmic poly(A)$^+$ mRNA level for LSAT-890 3′ indicates that greatly lengthening the distance between the poly(A) tail and the ARE does not alter the rapid turnover of the mRNA. Insertion of a long intervening segment in the normally stable LSGC mRNA between the stop codon and control $(AUUGC)_5$ element also did not alter its steady-state level, as expected (Fig. 8). Insertion of a 1.5-kb RNA fragment between the control $(AUUGC)_5$ element and the poly(A) tail also did not alter mRNA stability (data not shown). From these data, it can be concluded that following translation of the coding region, activation of the ARE can be enacted over long intervening lengths in the 3′ NCR.

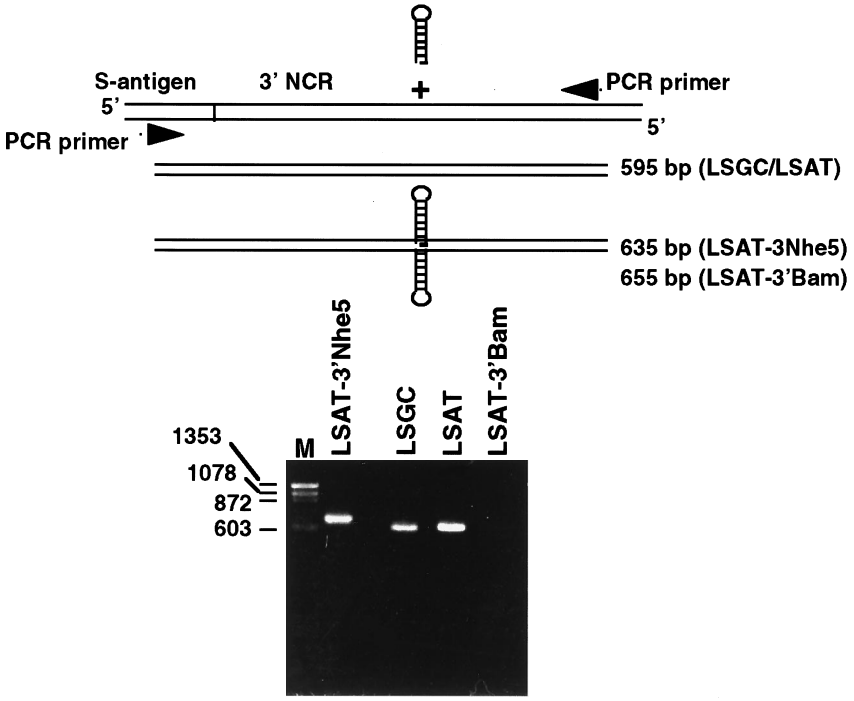

FIG. 6. Strong secondary structure (−80 kcal/mol) but not weaker structure (−30 kcal/mol) in the 3′ NCR blocks processivity by RT-PCR. HeLa cells were transfected with plasmids, whole cytoplasmic RNA was prepared, and equal amounts were subjected to RT with reverse transcriptase and a 3′ NCR DNA primer. cDNAs were then amplified by PCR with the primers shown above the gel (arrows) and resolved by agarose gel electrophoresis, and ethidium bromide-stained DNA was photographed. Successful RT-PCR of LSGC mRNA, of LSAT-3′Nhe5, and of LSAT-3′Bam should give rise to 595-, 630-, and 645-bp fragments, respectively. Lane M, DNA molecular size markers (in base pairs).

DISCUSSION

This study demonstrates that rapid decay of mRNAs containing the GM-CSF ARE is triggered by an activity that is coupled to translation of the short-lived mRNA and scans it processively in a 5′ → 3′ direction. This study also provides evidence consistent with the interpretation that the 3′ NCR must be accessible to a 5′ → 3′ processive event that activates mRNA degradation. That a 5′ → 3′ processive event is essential for activation of mRNA decay was shown by the ability of strong secondary structure to block rapid mRNA turnover when inserted at any position 5′ to the ARE but not when inserted 3′ to the element (Fig. 1, 3, and 4A). Strong secondary structure completely blocked degradation of the normally short-lived ARE mRNAs, because no evidence was found for accumulation of degradation intermediates (from either the 5′ or 3′ end) stalled at the site of stable secondary structure (Fig. 7). Although it is not known whether strong secondary structure (−80 kcal/mol) would stall the processivity of a 5′- or 3′-directed exonuclease in this system, less stable structures do so in *Saccharomyces cerevisiae* by a similar approach (12, 34). It is clear that translation of the coding region per se is not the 5′ → 3′ processive event that induces destabilization of the test mRNAs. Secondary structure in the 3′ NCR did not impair translation of the reporter protein but did stabilize the mRNA (Fig. 4A and B). Thus, activation of the GM-CSF ARE in this system involves a processive event that requires translation of the coding region to activate the downstream ARE in the 3′ NCR.

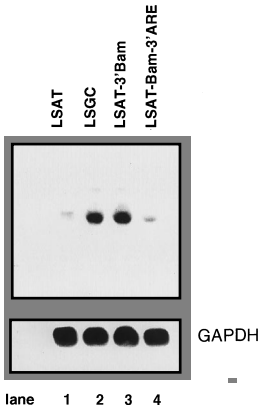

FIG. 7. Characterization of stable and unstable ARE mRNAs containing strong secondary structure in the 3′ NCR. HeLa cells were transfected with plasmids, whole cytoplasmic RNA was prepared, and equal amounts were analyzed by Northern blot procedures with a ^{32}P-labeled probe prepared from the entire S antigen coding region. The slightly retarded mobility of LSAT mRNA in lane 1 is a gel loading artifact and not reproducible.

It is possible but unproven that ribosomes, ribosomal subunits, or translation-related factors might constitute the activating signal that triggers rapid decay of mRNAs containing the GM-CSF ARE. The amount of secondary structure necessary to block *cis* activation of the GM-CSF ARE corresponds to that which also blocks ribosome transit (>−40 kcal/mol). Lesser amounts of structure (−20 to −30 kcal/mol) did not stabilize experimental mRNAs containing the GM-CSF ARE (Fig. 5) and did not block translation when inserted in the coding region (data not shown). On the other hand, strong secondary structure 5′ to the ARE may block the propagation of conformational or compositional changes to the 3′ NCR RNP that might result from translation of the 3′ terminus of the coding region rather than entrance of translation-linked factors into the 3′ NCR. It seems unlikely that the processive

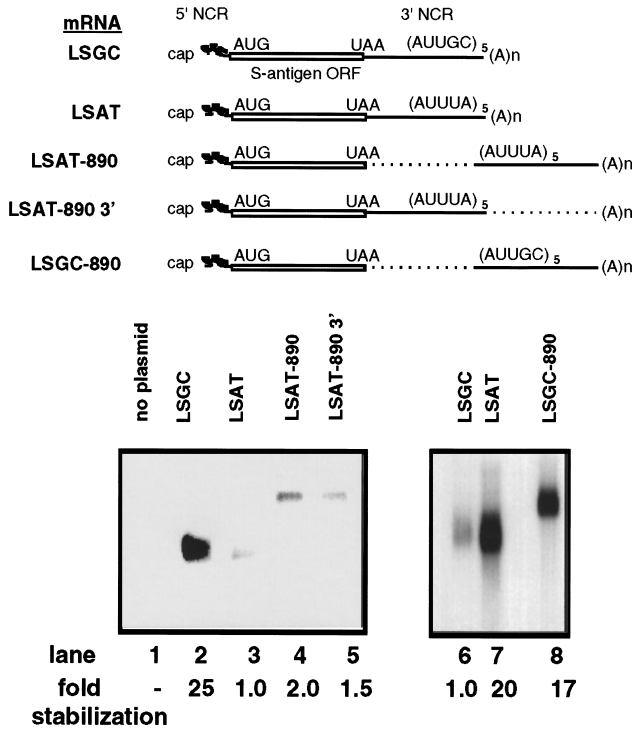

FIG. 8. Effect on mRNA stability of the intersegment length between the stop codon and the ARE and between the ARE and the poly(A) tail. An 890-nt fragment devoid of AU-rich and U-rich motifs and AUG codons was created. The fragment was inserted between the translation termination codon and ARE (LSAT-890 and LSGC-890) or the ARE and poly(A) tail (LSAT-890 3′). Northern analysis was performed on equal amounts of poly(A)$^+$ mRNA prepared 48 h after transfection. Quantitation of autoradiograms was carried out as described in the legend to Fig. 1.

event blocked by secondary structure directly corresponds to the movement of a 5′ → 3′ exonuclease, because there was no evidence for accumulation of decay intermediates degraded to the site of secondary structure.

More than half of cytokine AREs are not proximal to the translation termination codon. It is not known precisely how far ribosomes or ribosomal subunits transit on the mRNA after termination of translation in vivo. In fact, it is not even known whether ribosomes or ribosomal subunits actually dissociate from the mRNA after translation termination in vivo or whether they continue to migrate but simply lose the ability to bind initiation factors that are necessary for reinitiation at a downstream AUG (reviewed in reference 19). Since ribosomes protect roughly 30 to 40 nt of the mRNA from nuclease digestion (24), at the very least, an ARE within 40 nt of the stop codon could in principle interact with a ribosome at the termination codon, the last position of a ribosome on mRNA that is reliably known. Whether ribosomes could contact AREs located hundreds of nucleotides downstream from the termination codon cannot currently be answered. Analysis of the 3′ NCR sequences of a variety of cytokine mRNAs indicates that there are roughly two groups of 3′ NCRs. In one group (~41% [7 of 17]), the intersegmental length between the stop codon and ARE is short (5 to 43 nt; average, 30 nt). Direct interaction between a ribosome at a stop codon and the destabilizing element (or factors located at the ARE) is formally possible for these mRNAs. However, the second group of mRNAs (~59% [10 of 17]) have long intersegmental distances separating the stop codon from the ARE (140 to 440 nt; average, 262 nt) and

TABLE 1. Analysis of cytokine ARE 3′ NCR structure

mRNA species[a]	Distance between ARE and stop codon (nt)	Positions (nt) of 3′ NCR start/stop codons[b]
Hu IL-2	40	None
Mu IL-2	43	None
Bo IL-2	42	None
Hu IFN-γ	26	None
Hu G-CSF	24	None
Mu BSF-1	31	None
Hu BSF-1	5	None
Hu GM-CSF	~200	44/ARE, 68/ARE, 108/148
Mu IL-3	~150	41/109, 48/ARE
Ra IL-3	~140	40/79, 47/ARE, 77/ARE
Mu GM-CSF	~200	60/90, 102/130
Hu IL-1β	~340	36/50, 120/140

[a] Hu, human; Mu, mouse; Bo, bovine; Ra, rat; IL-2, interleukin-2; IFN-γ, gamma interferon; BSF-1, B-cell stimulatory factor 1.
[b] None, start/stop codons not present.

average eight to nine times longer than the first group. It is unlikely that downstream reinitiation generally takes place at a second open reading frame because it is an exceedingly rare event following long open reading frames, even at low levels, and few of these mRNAs contain intervening AUGs in good context and at an appropriate distance (see Table 1 for analysis). There is also no evidence for additional degradative signals in this class of mRNAs, since comparison of the 3′ NCRs does not reveal any sequences in common other than the ARE (unpublished observations). Thus, it is extremely unlikely that degradation of most natural cytokine mRNAs requires that a ribosome at the termination codon and the ARE directly interact over a short intervening distance (although looping of the RNA is still possible). Additionally, studies presented here with experimental mRNAs in which the GM-CSF ARE was separated from the stop codon by more than 1,000 nt (Fig. 8) displayed no increase in stability compared with parental mRNAs containing about 200 nt of intervening sequence.

What is the role of translation in rapid mRNA decay controlled by the GM-CSF ARE? There are some similarities between rapid decay of mRNAs mediated by the GM-CSF ARE and the mechanism of nonsense-mediated mRNA decay. Nonsense-mediated mRNA decay in *S. cerevisiae* is activated by premature translational termination (17, 40), which is thought to involve the continued scanning of some ribosomes, ribosomal subunits, or associated factors into the 3′ NCR, and their interaction with a downstream destabilizing element (61). In both systems, the process is cotranslational and requires the *cis* translation of the mRNA coding region to cause its destabilization. However, in nonsense decay in *S. cerevisiae*, mRNA degradation appears to occur processively in a 5′ → 3′ direction (17). In our study, degradation of ARE mRNAs to the point of secondary structure was not observed from either the 5′ or 3′ direction. In addition, primer extension analysis of the 5′ ends of the short-lived mRNAs did not detect differences in terminal lengths, indicative of decapping (data not shown), which is observed in nonsense-mediated decay (17). Nevertheless, if unstable mRNAs containing the GM-CSF ARE are turned over by decapping and 5′ → 3′ exonucleolytic degradation, it could be a more rapid or tightly linked process in mammalian cells than in yeast cells and intractable to the type of analyses used here. It is also possible that degradation of the RNA proceeds from multiple sites, accounting for the inability to recover decay intermediates stalled by secondary structure. Regardless, it is evident that in both nonsense-mediated mRNA

decay and rapid turnover triggered by the GM-CSF ARE, activation of mRNA degradation is cotranslational and involves processivity that extends 5′ → 3′ into the 3′ terminus of the coding region, and possibly the 3′ NCR, in an unknown manner.

If direct contact between the ribosome and ARE is required to activate mRNA decay, it is possible that a transiting ribosome could displace potentially protective binding proteins, such as AUBF (15, 42), making the RNA vulnerable to degradation. In contrast, changes in RNP structure may not require physical dissociation of protective proteins by transiting ribosomes but merely entry of the ribosome into the 3′ terminus of the coding region or the 3′ NCR. Strong secondary structure upstream of the ARE could conceivably block both its direct contact with 5′ → 3′ decay factors and propagation of structural changes into the 3′ NCR that might alter the conformation or composition of RNPs. Future studies need to concentrate on clarifying the events which occur after the termination of translation that affect activation of rapid mRNA degradation by ARE destabilizing sequences.

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